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NOTES

Ultrastructural-Immunohistochemical Evidence for a Maturation Defect of Temperature-Sensitive G31 Vesicular Stomatitis Virus in Murine Spinal Cord Neurons

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Ultrastructural immunoperoxidase studies were done in spinal cords of mice infected with wild type vesicular stomatitis virus or its temperature-sensitive (*ts*) mutant G31. Infected neurons showed subplasmalemmal staining of viral antigen and staining of viral particles budding from the neuronal membrane in wild-type vesicular stomatitis virus infection, whereas diffuse membrane and cytoplasmic staining with no budding virus was observed in *ts* G31 infection. Such findings suggest rapid viral assembly and release of viral particles from cells infected with wild-type virus. In contrast, maturation of *ts* G31 appears defective, and this would lead to accumulation of viral antigen in the cytoplasm of infected cells. These results correlate with studies in neuroblastoma cells which investigated the growth cycles of wild type, *ts* G31, and the spinal cord isolate of *ts* G31 as well as the viral protein-synthetic capacity of these viruses.

We previously reported that wild type (WT) vesicular stomatitis virus (VSV) produced a rapidly fatal 2- to 3-day illness in Swiss mice characterized pathologically by the development of large viral inclusion bodies in anterior horn neurons of the spinal cord (2). Ultrastructural studies of these neurons have shown active maturation of the virus by budding from the neuronal plasma membrane (2). In contrast, a temperature-sensitive (*ts*) mutant of VSV, G31, produces a slower disease which begins with hind limb paralysis at about 5 days and kills the animals in 5 to 9 days (8). Such disease is characterized pathologically by a peculiar status spongiosus of the spinal cord gray matter and by the absence of viral inclusions in neurons (1). These cells often show mitochondrial swelling and vacuoles in their cytoplasm. Because *ts* G31 VSV was capable of both altering the pathogenesis of VSV central nervous system infection and producing unusual morphological changes in infected animals, it was thought useful to assess its maturation in vivo.

Virological studies demonstrated rapid growth of WT VSV in brains and spinal cords of animals infected with 10^3 plaque-forming units (PFU) of WT virus. By 48 h after inoculation, 10^7 to 10^8 PFU/ml were detected in brain and spinal cords of mice infected with WT VSV. In contrast, animals infected with 10^4 PFU of *ts* G31 VSV demonstrated peak viral titers of only 10^5 to 10^6 PFU/ml of brain and spinal cord (8). Virus isolated from both the brains and spinal cords of *ts* G31 VSV-infected mice was shown to be exquisitely temperature sensitive (4).

To focus on the nature of intraneuronal events in *ts* G31 VSV assembly and maturation, we used ultrastructural immunohistochemical techniques on infected spinal cords. In addition, we have attempted a preliminary correlation between recent biochemical data obtained with infected neuroblastoma cells (4) and the ultrastructural studies reported here. These studies support the hypothesis that a specific defect exists in vivo in maturation and assembly of *ts* G31 VSV in neurons of infected spinal cords.

Three- to 4-week-old outbred Swiss mice, obtained from Scientific Products (Arlington Heights, Ill.), were injected intracerebrally with 2×10^3 PFU of WT VSV or with 10^4 PFU of *ts* G31 VSV under light ether anesthesia, as previously described (8). Animals injected with WT VSV were sacrificed in a moribund state 3 days postinjection, whereas mice injected with *ts* G31 VSV were sacrificed 5 days postinjection. This choice of time was based on previous experience;

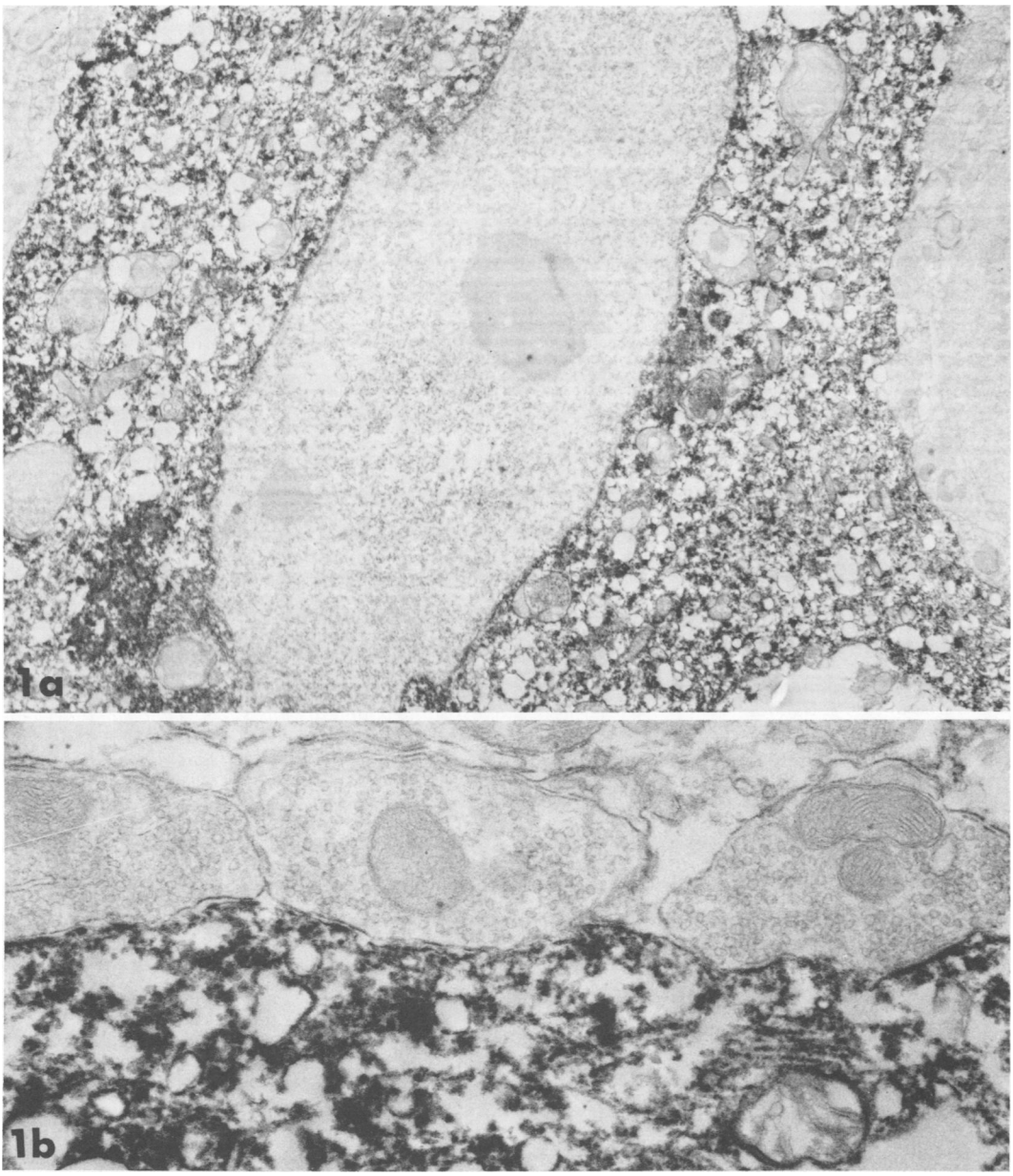

FIG. 1. *Electron micrographs of spinal cord neurons from mice infected with ts G31 VSV. Sections were reacted with anti-VSV IgG and incubated in PrA-P conjugate followed by diaminobenzidine. No counterstains were used. (a) Diffuse cytoplasmic staining is observed without subplasmalemmal antigen accumulations or viral budding. Such a picture is consistent with diffuse accumulation of nonstructural viral antigen in the cell because of inability by the ts mutant to assemble and mature. Mitochondria and cell nucleus are clean. ×7,500. (b) Higher-power photograph showing the peripheral portion of a neuron and three synapsing axon terminals. Diffuse staining of viral antigen in the neuronal perikarion contrasts with lack of staining in these terminals. ×30,000.*

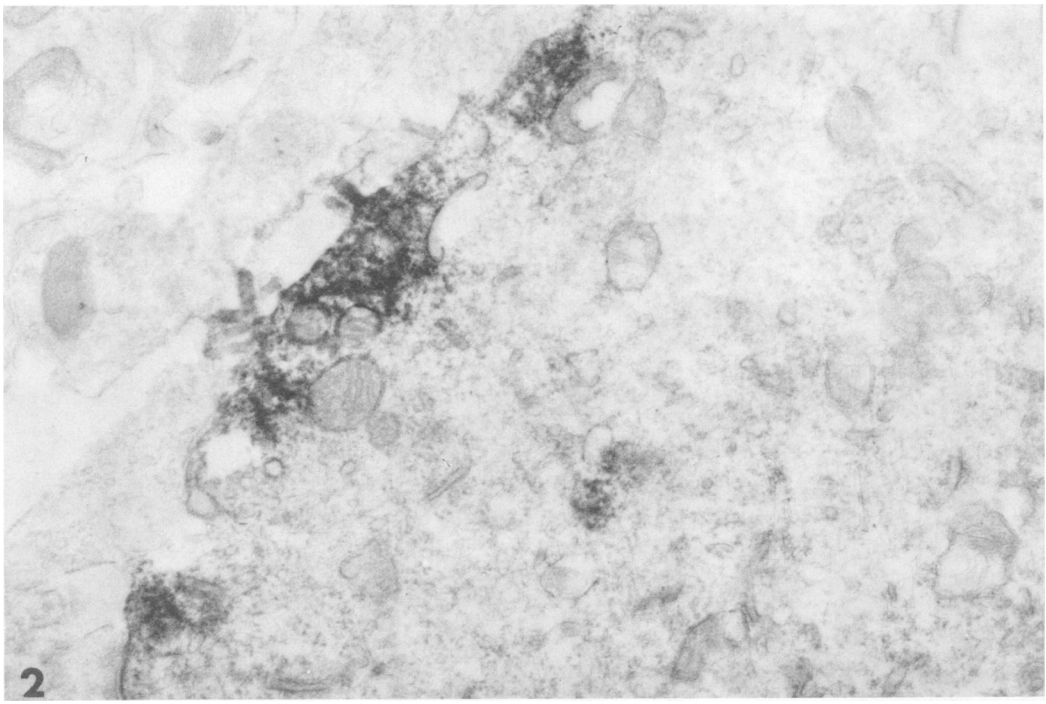

FIG. 2. *Electron micrograph of a spinal cord neuron from a mouse infected with WT VSV. The section was treated as in Fig. 1. Budding VSV particles are specifically stained, and distinct subplasmalemmal accumulations of viral antigen corresponding to the maturation sites are also evidenced. A small amount of reaction product is also seen in the cytoplasm inside a vesicle. It is, in fact, known that VSV may also mature at the level of intracytoplasmic vesicles. Note clean mitochondria. ×37,000.*

i.e., mice injected with WT virus showed maximum viral maturation between days 2 and 3 of infection, whereas mice infected with *ts* G31 VSV showed the status spongiosus best at around 5 days postinjection (1). During the entire course of *ts* G31 infection, intact virions were seen very rarely, and no viral budding from cellular membranes was observed (1).

Animals were sacrificed by total-body perfusion through the left cardiac ventricle with a 2% formaldehyde solution in 0.1 M cacodylate buffer at pH 7.3. Spinal cords were cut into segments of approximately 3 mm in length and left in fixative for an additional 3 h. After being washed in cacodylate buffer, spinal cord segments were sectioned in a Sorvall TC-2 tissue sectioner at 25-μm thickness and incubated overnight in purified anti-VSV immunoglobulin G (IgG) in dilutions from 1:10 to 1:40. The anti-VSV serum was prepared against whole virions and, as reported previously (8), possessed a neutralizing titer of 1:64,000. After sections were washed, they were incubated in a conjugate of protein A (Pharmacia) and horseradish peroxidase VI (Sigma) (PrA-P) at several dilutions from 1:50 to 1:300. (Protein A from the cell wall of *Staphylococcus aureus* binds to the F_c portion of mammalian IgG subclasses.) It was shown by Dubois-Dalq et al. (3) that use of PrA-P conjugate enhances the specificity of immunolabeling since less background activity is present than with peroxidase-labeled IgG. In addition, this method proved to be very sensitive, with strong antigen staining, even at dilutions as high as 1:250. After washing, sections were reacted in the substrate diaminobenzidine in the presence of H_2O_2, postfixed in 2% glutaraldehyde for 1 h, osmicated, and processed for Epon embedding. Ultrathin sections were cut without previous thick sectioning to examine the superficial layers of tissue which showed the best antiserum and conjugate penetration. Grids were studied without counterstains in a Philips 200 electron microscope.

The following controls were used to assure the specificity of the immune reaction. Sections from BHK-21 cells infected with Theiler's GD VII virus and showing large amounts of viral antigen by immunofluorescence were reacted with anti-VSV IgG and anti-GB VII IgG before PrA-P incubation; they only stained with the latter. Sections from brains of mice injected with WT

VSV or *ts* G31 VSV were incubated with anti-GD VII IgG before PrA-P incubation and showed no staining. Sections from brains of mice injected with WT VSV or *ts* G31 VSV were incubated with PrA-P without previous treatment with the antiserum and showed no staining. Sections from the WT or *ts* G31 VSV-infected animals were treated with the anti-VSV serum and then reacted with diaminobenzidine without PrA-P incubation; these sections showed no staining. Finally, spinal cords from uninfected mice failed to demonstrate any staining with PrA-P.

For studying the growth of *ts* G31 VSV in neuroblastoma cells, N-18 cells were obtained from Brian Spooner, Kansas State University, and a density of 4×10^6 cells per ml was infected with *ts* G31 VSV at a multiplicity of infection of 10. The virus was allowed to absorb for 15 min at 25°C, and then the cells were washed by the addition of 10 ml of Hanks balanced salt solution and pelleted by slow-speed centrifugation. The cells were washed twice more, diluted to 10^5 cells per ml, and then dispensed to small tubes which were incubated in a water bath maintained at 31, 37, or 39°C. At various times, tubes were removed from incubation and frozen at −85°C until the yield of infectious virus was determined with BHK-21 monolayers incubated at 31°C (4). The same procedure was used when neuroblastoma cells were infected with virus isolated from the spinal cord (*ts* G31 SC) of Swiss mice infected with *ts* G31 virus.

Cellular details were well preserved in both *ts* G31 VSV- and WT VSV-infected tissues, and viral antigens were stained by the PrA-P technique in both infections. Neurons infected with *ts* G31 VSV only showed membrane and cytoplasmic distribution of antigen without evidence of viral components accumulating in subplasmalemmal position and without any budding of virus (Fig. 1a and b). Diffuse antigen distribution and lack of viral budding would be consistent with little or no viral maturation and consequent cytoplasmic accumulation of viral antigen. Neurons of spinal cords infected with WT VSV, on the other hand, showed distinctive patches of positive staining in the subplasmalemmal cytoplasm, and, in addition, positively stained budding virions were observed in such areas (Fig. 2). Such findings suggest that assembly and release of WT virus from infected cells into the extracellular space occurs within 48 h, in contrast to the defective intraneuronal maturation of *ts* G31 VSV.

Studies were next performed to determine the yield of infectious virus in neuroblastoma cells infected with WT VSV, *ts* G31 VSV, and the viral isolates from *ts* G31-infected spinal cord (*ts* G31 SC). Whereas neuroblastoma cells infected with WT VSV gave similar yields of virus at 31, 37, and 39°C (Fig. 3), cells infected with *ts* G31 VSV (Fig. 4) gave a high yield of virus at 31°C, a reduced yield at 37°C, and no growth of infectious virus at 39°C. Cells infected with *ts* G31 SC showed brisk viral replication only when incubated at 31°C (Fig. 5). At 37°C *ts* G31 SC maturation only began at around 9 h after a prolonged period of viral eclipse. As with *ts* G31, no infectious virus synthesis was detected at 39°C in neuroblastoma cells infected with *ts* G31 SC. These results support the immunohisto-

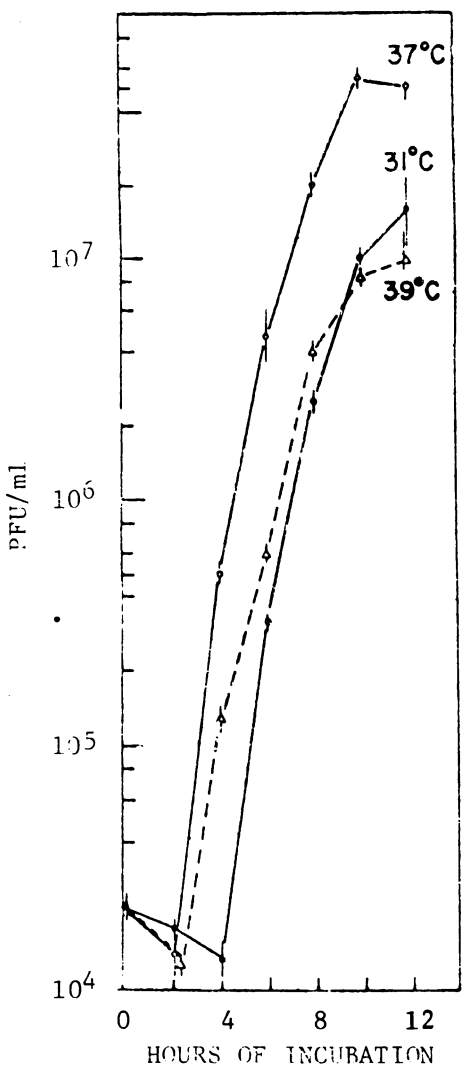

FIG. 3. *Growth of WT VSV in neuroblastoma cells. Cells give similar yields of virus at 31, 37, and 39°C.*

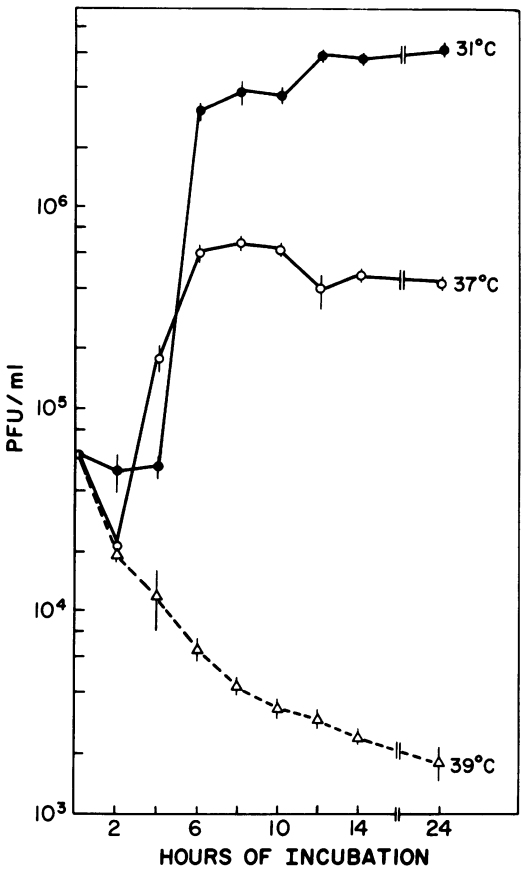

FIG. 4. *Growth of ts G31 in neuroblastoma cells. Cells give high yield of virus at 31°C, a reduced yield at 37°C, and no growth of infectious virus at 39°C.*

chemical studies reported above demonstrating lack of maturation and assembly in spinal cords of mice infected with *ts* G31 VSV.

Finally, viral protein synthesis of *ts* G31 VSV and *ts* G31 VSV SC were studied in neuroblastoma cells in culture. The assembly of the major structural proteins, labeled with [^{35}S]methionine, was quantitated by measuring the distribution of the proteins in the cytosol and membrane fractions of infected neuroblastoma cells (4). For the WT VSV, N, M, and G proteins were incorporated into the membrane fractions almost totally, suggesting that the viral proteins were being assembled at the plasma membrane (at 31, 37, and 39°C). In contrast, in the case of *ts* G31 and *ts* G31 SC, although the viral proteins were associated with the membrane fraction at 31°C, at the nonpermissive temperature (39°C) a significant amount of the M and N proteins remained in the cytosol fraction. At 39°C the G protein appeared to be incorporated into the cell membrane, suggesting that the *ts* G31 virus was not capable of synchronous maturation at the nonpermissive temperature (4). Other members of complementation group III, like *ts* G31, have also been associated with defective synthesis and/or maturation of the M protein (5–7) at nonpermissive temperatures. The M protein is thought to have an important role in the assembly of the virus and apparently is responsible for the binding of the ribonucleocapsid material to the viral envelope, which may explain why the assembly of both the M and the N proteins was altered at 39°C (4). Since it appears that the synthesis and maturation of the M protein, as well as that of the N protein, is affected in *ts*

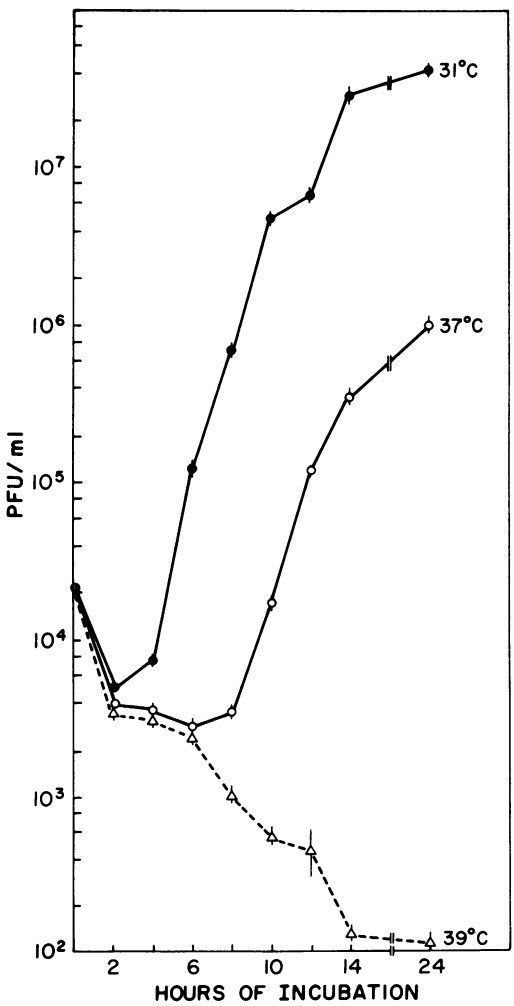

FIG. 5. *Growth of ts G31 SC in neuroblastoma cells. Viral replication is brisk at 31°C, begins after prolonged viral eclipse at 37°C, and is lacking at 39°C.*

G31, it may well be that an altered assembly of the virus in the spinal cord of an animal may underlie the mechanism responsible for the spongiform lesions produced by *ts* G31 virus.

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